AN ABSTRACT OF THE THESIS OF

Sjahrul Bustaman for the degree of Master of Science in Fisheries Science presented on January 14, 1992.

Title: Influence of Water Hardness on Gill Accumulation and Acute Toxicity of Aluminum in Rainbow Trout

Abstract approved: Redacted for Privacy
Lawrence R. Curtis

Rainbow trout were exposed to aluminum at pH 7.25 and 8.25 and four hardnesses (10, 30, 80, and 120 ppm $CaCO_3$) for 96 hours in a continuous-flow system and mortality and aluminum accumulation in the gills were determined.

Temperature, pH, and dissolved oxygen were measured daily for each treatment. Dissolved and total aluminum concentrations and hardness were determined following exposure periods of 48 and 96 hours. Aluminum was most toxic at pH 8.25, and was more toxic at lower than at higher hardnesses. Water hardness provided a significant protective effect against aluminum-induced mortality ($p < 0.05$), and there were no significant effects for water hardness on gill accumulation at either of pH. At pH 7.25 no mortalities occurred under any conditions. At pH 8.25, the accumulation of aluminum in gill tissues was higher than for pH 7.25 following exposure for 96 hours.

In addition, aluminum concentration and exposure time had a significantly cumulative effect on fish mortality ($p < 0.05$).

Possible mechanisms for aluminum toxicity and the accumulation of aluminum in the gills of rainbow trout were attributed to the forms and solubilities of aluminum species at different pH values. Competition between $Ca^{2+}$ and aluminum for binding sites on the gills likely influenced aluminum toxic action.

# Influence of Water Hardness on Gill Accumulation and Acute Toxicity of Aluminum in Rainbow Trout

by

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A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Master of Science

Completed January 14, 1992

Commencement June 1992

APPROVED:

Redacted for Privacy

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Date thesis is presented January 14, 1991

Typed by B. McMechan for Sjahrul Bustaman

Acknowledgements

I would like to thank Dr. Larry Curtis for providing me with the opportunity to work with him, and for his valuable advice during my course of study. Without his guidance and aid, I would have been unable to complete this course of study.

I would also like to thank Mr. Wayne Seim for his helpful advice, particularly for his concept of modeling research equipment, Dr. John Westall for his assistance with chemistry problems, and Dr. Henry A. Froehlich, who served as the graduate representative on my committee.

Grateful thanks are also extended to Deke Gundersen and Katherine Super for their laboratory assistance, to Quan Zhang for his help with computers, and to Susan Allen for her assistance with the English language. I would also like to extend my general gratitude to all of the staff members who helped me at the Oak Creek Laboratory during the completion of this research.

Finally, I would like to thank my wife, Nurhasanah, and my sons, Renaldy and Filardy, who provided me with support, patience and love during my course of study.

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# Influence of Water Hardness on Gill Accumulation and Acute Toxicity of Aluminum in Rainbow Trout

## 1. INTRODUCTION

Trace amounts of heavy metals can result in adverse biological reactions and these substances (e.g., cadmium, mercury and copper) have been studied extensively (Howarth and Sprague, 1978;Olson et al., 1973;Part and Svanberg, 1981;Rodgers and Beamish, 1981). On the contrary, aluminum has received less attention, although aluminum is the most common metal within the crust of the earth. In most natural waters, concentrations of aluminum are less than one part per million (ppm). However, aluminum concentrations above natural levels are commonly caused by mining activities and acid precipitation (Freeman and Everhart, 1971). Varied conditions which can contribute to high aluminum concentrations in waters associated with mining include release of process effluents, leaching from exposed pyritic surface ores in surface operations, as well as releases from smelting and refining operations.

Many other factors besides pH and ligand as well colloid stability, dissolution or precipitation kinetic , crystal growth influence species of aluminum. The

chemistry of aluminum in surface waters is complex because of the five following properties (Campbell et al., 1983; Hem, 1968; Hem and Roberson, 1969; Hsu 1968; Smith and Hem 1972: cited in U.S. Environmental Protection Agency [USEPA], 1988):

1) Aluminum is amphoteric and more soluble in acidic or basic solutions than in neutral solutions;
2) Chloride, fluoride, phosphate and sulfate ions form soluble complexes with aluminum;
3) Aluminum can form strong complexes with fulvic and humic acids;
4) Hydroxide ions combine with aluminum ions to form soluble and insoluble polymers; and
5) Under certain conditions, aluminum solutions in water are slow to approach chemical equilibrium.

At low pH levels, aluminum solubility, which is associated with toxicity to fish and other aquatic organisms, increases (Haines, 1981). In acidic environments, soluble forms of aluminum are polymeric and cationic ($Al^{3+}$), while monomeric anions [$Al(OH)_4^-$] are present in basic media and insoluble hydroxide, $Al(OH)_3$, in the pH range 6.5—7.5 (Hunter et al, 1980).

The increase in aluminum solubility which occurs under alkaline conditions has been considered as important determinant of toxicity in rainbow trout (Freeman and Everhart, 1971). Many experiments have demonstrated that

toxicity is directly related to soluble aluminum concentrations.

Freeman and Everhart (1971) reported the time to 50 percent aluminum-induced mortality for rainbow trout at various pH levels. They determined that toxicity among rainbow trout increased sharply with increases in pH levels. At pH 7.0—8.0, mortality caused by 5.2 ppm aluminum did not exceed 30 percent for a 45-day period, but 50 percent mortality occurred within 8 days at pH 8.5; and at pH 9.0, 50 percent of the fish died in 2 days. The water quality for pH range of 6.5 to 9.0 appears to adequatly protect fresh water fishes and bottom dwelling invertebrate fish food organisms from effects of the hydrogen ion (U.S. EPA 1976 in U.S. EPA 1988). Moreover, since the pH of water changes as it passes the gills of fish, any environmental contaminant for which toxicity varies with pH may be more or less toxic at the gills than would have ordinarily been predicted from bulk water pH (Playle and Wood, 1989).

The mechanism of aluminum toxicity remains an unknown. However, there are three possible routes by which aluminum in water can be absorbed by fish : the gills, the body surface, and the alimentary canal. It is likely that the gills are the most important route for the absorption of aluminum when fish are exposed to high concentrations.

Teleost fish possess four pairs of gill arches. There are double rows of gill filaments on each arch that branch

out from one point, like a letter Y (Figure 1.1). In rainbow trout, the epithelium of the secondary lamellae consist of two layers of cell joined by desmosomes and tight junctions (Figure 1.1). Water flows from the outer to the inner side of each filament, passing between the secondary lamellae and into the space beneath the operculum, from which it exits the gill chamber.

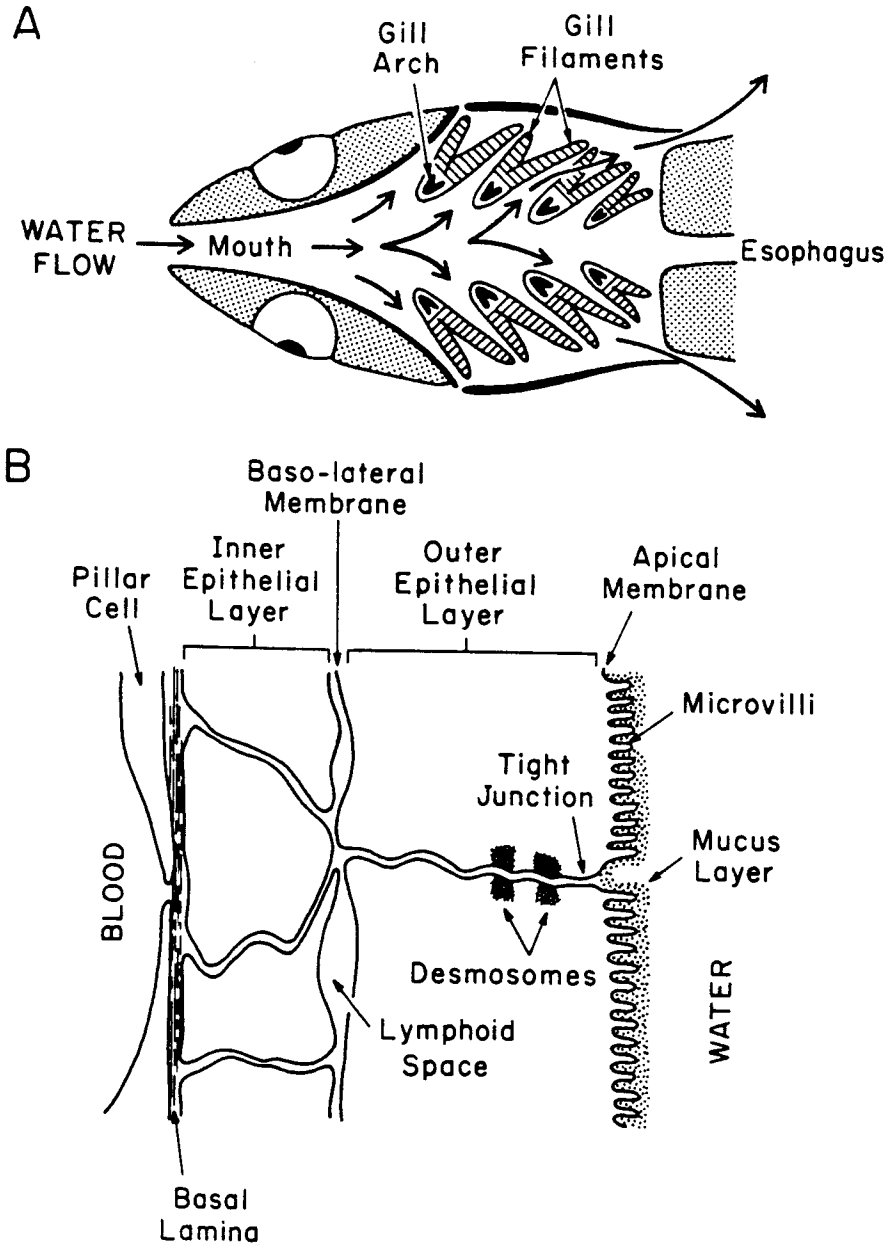



Figure 1.1. Structure of a rainbow trout gill (Mc Donald, 1983): A) Horizontal section trough the head showing gill arches filaments and direction of water flow. B) Cross section through the lamellar epithelium showing cell layers, in the outer layer the cells are bound together by apical tight junctions and by desmosomes.

The absorbtion rate of toxicant by the fish gill depends upon the concentration of the toxicant and the rate of movement of water across the secondary lamellae. High flow rates present greater amounts of the toxicant to the gill surface for each unit of time exposure. Also the rate of uptake depends upon the penetration of the toxicant across the multiple cell layers (Figure. 1.1). The physicochemical properties of the toxicant lipid solubility, size and charge largely determines its permeability to this barrier. In general, the main target organ appears to be the gills, as in the case with $H^+$ toxicity (Buergel and Solturo, 1983).

Booth, McDonald, Simons and Wood (1988) postulated two distinct possible mechanisms for aluminum toxicity, the first based on solubility and the second based on speciation. Schofield and Trojnar (1980) suggested that aluminum toxicity was a function of the transformation of soluble monomeric aluminum species into either polymers and/or precipitates at the gill surface. This precipitation results in gill damage and subsequent osmoregulatory disturbances. In the second possible mechanism, based on differences in toxicity among aluminum species, the basic assumptions were that soluble aluminum species vary in terms of their reactivity with surface binding sites on the

gills, and polymerization and/or precipitation are secondary events in toxicity. However, these two potential toxic mechanisms are probably not mutually exclusive, both actively produce gill damage, and the predominant mechanism may vary with specific conditions. Factors determining the predominant mechanism of toxicity could include pH, ionic calcium ($Ca^{2+}$) concentrations, or the duration of exposure if there are major adaptive changes occurring at the surface of the gills.

The biological action of $Ca^{2+}$ is often dependent upon its ability to bind to proteins, and is related to its basic chemical properties. Calcium binds to the surface of the gills, a process which is essential for the maintenance of the ionic and osmotic gill regulatory functions. Competition for binding sites on the gill between $Ca^{2+}$ and other divalent or trivalent metallic ions may influence metal uptake and resultant toxicity to fish (Hunn, 1985).

At acidic pH levels, $Ca^{2+}$ protects against aluminum-induced ion loss, thereby reducing the mortality of brook trout (Booth et al., 1988). Furthermore, $Ca^{2+}$ is similarly antagonistic to toxicity when low pH is a singular factor. This level of protection is believed to arise from weak ionic interactions between $Ca^{2+}$ and surface ligands (i.e., membrane integral and peripheral proteins, mucopolysaccarides, and intercellular anionic residues). These interactions act to stabilize the apical membranes of the gill epithelium, increasing the tightness of the intercellular

junctions (McDonald, 1983). This may reduce membrane permeability and increase its resistance to attack by surface-active toxicants. In these ways, increasing water $Ca^{2+}$ may delay or reduce the binding of aluminum to the gill. Alternatively, high water $Ca^{2+}$ may promote the polymerization or precipitation of aluminum to gill surfaces since neither of these processes requires the prior binding of aluminum to gill anions.

Mucous accumulation and gill necrosis are among the reported reactions of fish exposed to elevated aluminum concentrations (Freeman and Everhart, 1971). Both effects are less severe at low pH levels (4.2—4.6) than for aluminum solutions at pH levels of 5.2—5.6 (Baker and Schofield, 1982). At times, however, mortality has occurred without visible gill damage. Both elevated aluminum concentrations and low pH levels have been reported to interfere with osmoregulation (Playle et al , 1989).

Dissolved and suspended forms of aqueous aluminum are moderately toxic to the rainbow trout, although signs of aluminum poisoning differ for the two toxicant forms. At 5.2 ppm of dissolved aluminum, extremely acute mortality occurred, whereas mortality rates were delayed with exposure to equivalent suspended amounts in acidic solutions(Freeman and Everhart, 1971). Effects on activity and coloration were also more pronounced in the dissolved concentrations.

Although the effects of acutely lethal and sublethal concentrations of aluminum have been determined in a number of studies, the ability to predict of lethal action remains highly uncertain in natural waters. For the rainbow trout, Call et al. (1984) reported mortality rates at pH 6.5, 7.5 and 8.5. At a nominal pH of 6.5 (i.e., a mean measured pH of 6.59), LC50 estimates for pooled replicate data at a 95 percent confidence interval for 96 hr of exposure was 7.4 (5.8—9.4) ppm; at a nominal pH of 7.5 (i.e., mean measured pH of 7.31), LC50 estimates for 96 hr was 14.6 (9.3—23.1) ppm; and at a nominal pH 8.5 (i.e., mean measured pH of 8.17), there were insufficient mortalities to determine an LC50 estimate. This wide range of aluminum toxicity suggests that chemical, physical and physiological factors may have important effects. In particular, the character of the surface water and its relation to the aluminum species should be considered. Two factors of apparently primary importance are water hardness and pH. Increased water hardness increases the resistance of fish to aluminum (Booth et al., 1988).

Behavioral observations and mortality rates indicate different modes of toxic action for dissolved and suspended aluminum complexes. The response to chronic exposure to suspended aluminum indicated a limited physiological effect. Loss of appetite and gill hyperplasia were initiated sooner at higher concentrations than at lower concentration.

Playle et al. (1989) resolved two toxic mechanisms for aluminum at acidic levels of pH: 1) Ionregulatory toxicity, caused by aluminum at pH 4.8 and 5.2, and by acidity at pH 4.4; and 2) respiratory toxicity, caused solely by aluminum which increased with increased pH. Higher water calcium reduced ionregulatory disturbances solely due to acidity, but not those due to aluminum at higher levels of pH; higher water calcium also reduced respiratory disturbances at lower pH levels, whereas this was not true of higher pH levels. To assess whether gill aluminum accumulation was correlated with physiological disturbances, Playle and his colleagues sampled gills from surviving fish at the end of some of the experiments. At all three acidities, gill aluminum concentrations were elevated in the presence of aluminum, while higher water $Ca^{2+}$ reduced gill aluminum accumulation. The mechanism by which $Ca^{2+}$ serves to reduce aluminum accumulation at the gills is unknown, but it has been hypothesized that $Ca^{2+}$ competes with aluminum for binding sites. The effect of $Ca^{2+}$ upon aluminum binding at the gills did not reduce the respiratory effect of aluminum for the pH 5.2 treatments, possibly since aluminum precipitation at this level may simply be too great to be ameliorated by the $Ca^{2+}$. Aluminum's respiratory toxicity at pH 4.8 was reduced by $Ca^{2+}$, but this was not true for ionoregulatory toxicity. Perhaps only a small amount of precipitated aluminum is required to cause gill inflammation. In this case, owing

to the swelling of cells and mucus accumulations, ion losses as well as larger amounts of precipitated aluminum would be necessary to impair the increased diffusion distance of the gas transfer trough.

The chemistry of aluminum and the interactions between $Ca^{2+}$ and biologically significant aluminum in acidic media are more clearly defined than those for basic media. It is of primary importance to understand the mechanisms of aluminum toxicity in basic media since aluminum speciation is clearly pH dependent.

In this study, accumulation of aluminum by rainbow trout gill in relation to acute mortality was investigated, considering water hardness and pH as the independent parameters.

# 2. MATERIALS AND METHODS

## 2.1 Experimental Equipment

To examine the influence of hardness on aluminum toxicity in rainbow trout, a large volume water supply with low dissolved solids was required to provide dilution water reconstituted at appropriate levels of hardness and aluminum concentrations. A reverse osmosis (RO) system, producing approximately 4 L/minute of water with total dissolved solids of approximately 4 mg/L, representing a reduction greater than 95 percent of comparable substances in feed waters, was selected and installed. Figure 2.1 presents a schematic view of the rainbow trout exposure apparatus. The RO system was used to supply water to four 1,000 gal fiberglass storage tanks in which , aluminum stock, dilution water stock, and high hardness stock solutions were mixed. The flow combinations from these four lines supplied the dilutor which provided four aluminum concentrations and a control solution at four levels of hardness for a total of 20 treatments. One pH level was tested for each experiment.

For experiments at a nominal pH of 8.25, the aluminum stock solution was maintained at pH 9.1, while the RO and high water hardness stocks were at pH 8.7. For experiments

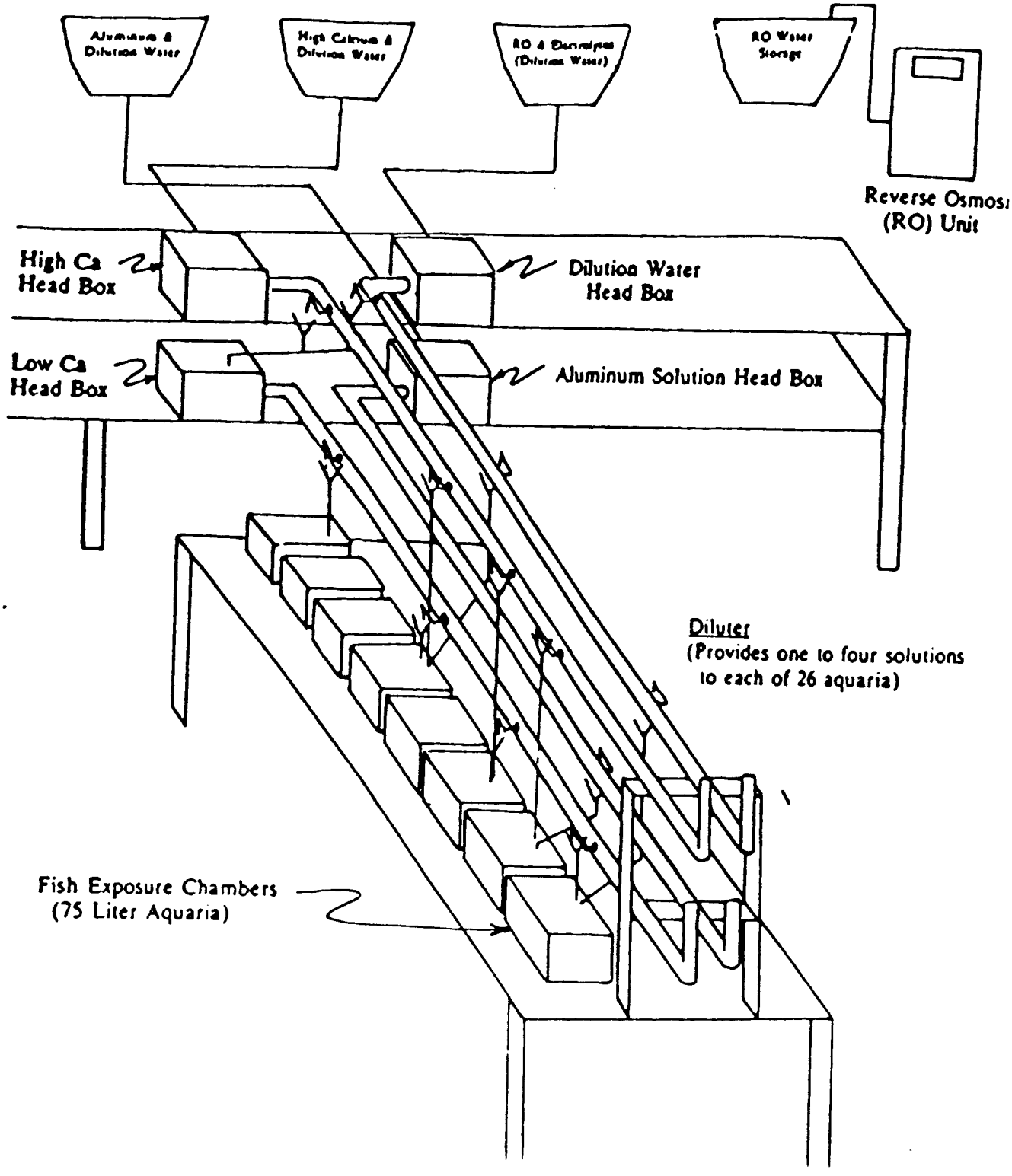



Figure 2.1. Schematic drawing of the rainbow trout exposure apparatus.

conducted at a nominal pH of 7.25, $CO_2$ gas was used to automatically maintain the aluminum solution at pH 7.0, with the RO and high water hardness stocks also at pH 7.0. The pH levels were adjusted with either 1N NaOH or 1N HCl. A continuous-flow Chadwick-type diluter was modified to mix the flows into aquaria of 75 liters in capacity containing 20 liters, and water temperatures were controlled at $14^0$C. Nominal flow rates were 100 ml/min for each aquaria resulting in a volume replacement time of 3.3 hours.

## 2.2 Acute Toxicity Test

Juvenile rainbow trout weighing from about 1 to 3 grams were subjected to 96 hr, flow-through exposures with varying hardness and aluminum concentrations at pH 7.25 or 8.25. There were 10 fish in each chamber and 20 fish for each concentration. The dissolved oxygen, temperature and pH of the exposure water were measured daily, while hardness and aluminum concentrations were measured after 48 and 96 hours of exposure. Aluminum concentrations in fish gills were determined by methods developed by Playle et al. (1989). Acute toxicity was analyzed using the trimmed Spearman-Karber method to determine the 96 hr LC50 values (Hamilton et al, 1977). A one-way analysis of variance (ANOVA) was used to compare gill aluminum concentrations for the varying degrees of hardness.

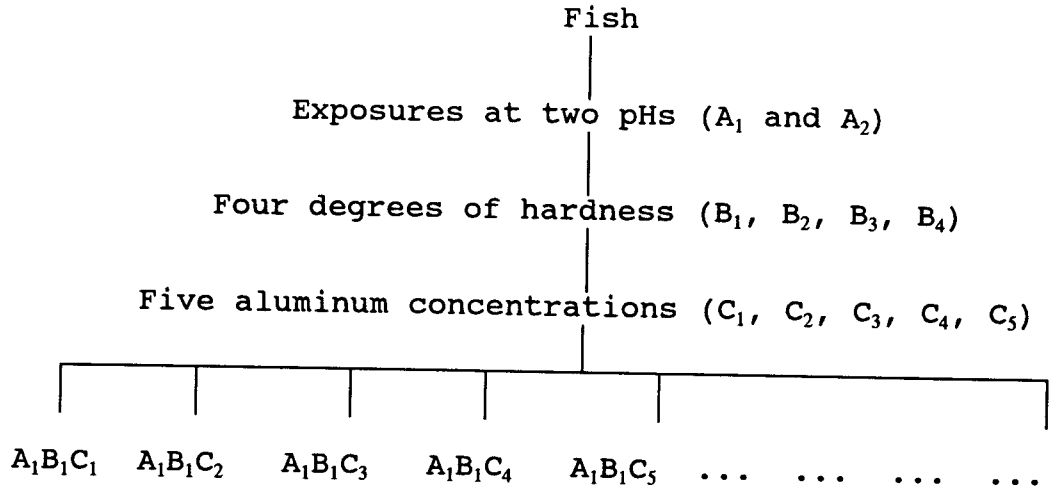
## 2.3 Analytical Chemistry

A photometric method was used to determined aluminum (total and dissolved )concentrations of exposure waters, employing catechol violet as a chromaphore (Dougan and Wilson, 1974). Samples (15-20 ml) were collected midway between the surface and the bittom of the water columnin the center of each aquaria. Dissolved aluminum defined as water sample of aluminum pass through 0.40 $\mu$m polycarbonate filter (Millipore) while total aluminum is aluminum concentration of aquaria's sample. Filtering vacum equipment (Millipore) was used to get dissolved of aluminum samples. Each dissolved sample (10 ml) needs one filter and about 30 second for filteration. Gill aluminum concentrations were determined by application of the method developed by Playle et al. (1989). After 96 hr for the surviving fish, and for some of the treatments at 24, 48, 72, and 96 hr for dead fish, a section of whole gill was removed from each fish for aluminum determination. Each gill sample was then placed in 15 mL of distilled water for 1 min to remove excess, loosely-bound aluminum, and then was frozen. Filaments were subsequently cut from the gill arches, weighed, and then digested in five times their weight of 0.05 M reagent grade $H_2SO_4$ for 8 hr at $80^0$C. The supernatant was analyzed for aluminum using the pyrocatechol violet method (Dougan and Wilson 1974).

A pH meter was used to determine pH values; the dissolved oxygen values were determined by an oxygen electrode and meter; and hardness values, expressed in mg $CaCO_3$/L, were determined by the EDTA titrimetric method.

## 2.4 Experimental Design

The flow chart for this research follows:




where

A = pH at $A_1$ = 7.25 and $A_2$ = 8.25;

B = hardness concentrations ($CaCO_3$) in ppm at $B_1$ = 10, $B_2$ = 30, $B_3$ = 80, and $B_4$ = 120; and

C = aluminum concentrations (ppm) at $C_1$ = 0, $C_2$ = 1, $C_3$ = 2, $C_4$ = 4, and $C_5$ =8;

and where the number of samples is 40, with each experiment carried out twice.

# 3. RESULTS

The pH variation within the experiments was 0.02 (mean measured pH 8.27 with an SD (standard deviation) = 0.2001) and 0.36 (mean measured pH 7.61 with SD = 0.0356) at, respectively, nominal pH levels of 8.25 and 7.25. The effect of pH on the solubility of aluminum is shown in Figure 3.1. At lower pH values, correspondingly lower amounts of aluminum were filterable.

The pertinent data for each test used for the calculation of the LC50 values are presented in Tables 3.1 and 3.2. Temperatures varied less than $\pm 1^0$C from the standard of $14.4^0$C during the tests. In treatments tested, the mean dissolved oxygen values during the experimental period were 9.96 ppm $O_2$ and 10.93 ppm $O_2$ at, respectively, nominal pH levels of 7.25 and 8.25. Dissolved oxygen decreased with increased aluminum concentrations for each water hardness treatment.

Aluminum (Al III) occurs in variety of forms in natural waters as illustrated in Table 3.3 (Nordstrom et al.,1990; Apps and Neil, 1990; Hem and roberson, 1990; May et al .,1979; Nordstrom and May, 1989; Bertsch, 1989). Among the many species indicated in the table, the species of primary importance (i.e., account for most of the Al(III) in these experiments are the hydroxide species, and possibly the complex organic species. All of the solutions

in this study appear to have been supersaturated with respect to $Al(OH)_3(s)$. Thus, among the hydroxide species, we envision a continuous maturation of simple Al(III) complexes ultimately to the crystalline form, with no clear distinction among the the polymeric, colloidal, and amorphous solid forms. We expect a relatively rapid ($\tau$ < minutes or hours) equiliration among the simple mononuclear species and progressivelly longer equilibration times to the stable crystalline ($\tau \geq$ days or weeks).

Unfortunately, solutions with these intermediate metastable colloidal species extremly difficult to characterize. There is no firm scientific basis from which:

1. to calculate a quasi steady state pseudo-equilibrium distribution among these poorly defined species;
2. to calculate the extent of each of these reactions as a function of time from kinetic equations;
3. to determine concentrations of these species analytically.

If we absolutely needed to know the Al(III) speciation in solution, we could

4. work under conditions at which the solution was not saturated with $Al(OH)_3$ (s) (e.g., low Al(III) concentrations, extreme pH values, or

high concentration of organic complexing agents), or

5. work with aged , saturated solutions, for which it could be confirmed that the intermediate metastable colloidal forms had ripened into non-suspended crystals.

However, these last two options are inconsistent with one of the conditions necessary for the toxicity tests (i.e., relatively high toatal concentrations of Al(III) in solution). Furthemore, the toxicities of these difficult-to- characterize intermediate species are of great interest themselves.

Therefore, the experiments were run at total concentrations of Al(III) that greatly exceed the solubility limit and attempt to gain from the data to what extent maturation of the Al-OH into crystals has occured. We used the standard (but crude) method of separating the Al(III) into two fractions:

(i) total Al(III) in solution and

(ii) Al(III) that which will pass through a 0.40 $\mu$m filter.

The latter fraction is often referred to as the "dissolved" fraction, but it undoubtedly contains colloidal particles tht are not "dissolved" in the true sense of the word; therefore, we refer to the fractions as "total Al(III) and "filterable Al(III)" consistent with the operational determination.

Our examination of the data was carried out into two steps: (i) comparison of filterable Al(III) to total AL(III), to determine whether the change in concentration of filterable Al(III) with that of total Al(III) resembles that of a solubility process or that of a partition process; and (ii) comparison of filterable Al(III) to soluble Al(III) calculated from an equilibrium model, to compare the absolute value of the degree of saturation. Both of these steps should tell us how far along the path to equilibrium with the crystalline solids the solutions are.

The examination of data at nominal pH 7.25. For the total Al(III), Figure 3.2 shows that the total Al(III) determined in solution tracks the nominal added values very well for both Trials I and II (8ppm = 296 $\mu$m).

However, for filterable Al(III), Figure 3.3 shows greatly different results for Trials I and II, the filterable Al(III) is reproducibly 10-12% of the total Al(III); such a distribution would be consistent with (i) slow first order kinetics for the removal of Al(III) by precipitation, (ii) complexation and solubilization of Al(III) by an organic complexing agent present in concentrations much greater than the concentration of Al(III) (e.g., mucous), or (iii) filterable Al(III) actually equal to total Al(III), but with some kind of systematic factor-ten error in the analytical procedure.

The time scale of the experiment certainly supports the lacks of equilibrium suggested in the first explanation. The Al(III) spiking solution is maintained at pH >9.0 before being spiked into the head box and the aquaria at pH ≈ 7.6; the solution remains at the lower pH for a maximum of about 18 hours. It would not be surprising if solubility equilibria are not approached in this interval. An interesting ancillary experiment for a future study would be to establish another aquarium with an extremely long residence time and follow the evolution of Al(III) concentrations.

The second explanation points up the desirability for DOC determination for selected experiments, or testing of the ability of mocuos to enhance the solubility of $Al(OH)_3$ (s) crystals.

In contrast to the data for Trial II, the data for filterable Al(III) in Trial I (Figure 3.3) is approximately independent of the total Al(III) added, as would be expected if Al(III) were in solubility equilibrium.

The data obtained for both trials at nominal pH 8.25, which are shown in Figure 3.2 and 3.3, reflect the same trends as described for nominal pH 7.25, Trial II: total Al(III) tracks nominal added Al(III) well, but with considerable scatter, and filterable Al(III) is about 10-11 % of total Al(III). The unlikelihood that the kinetics of precipitation or the binding by organics would be independent of pH over this range challenges the first and

second explanation for the 10-11% offred above for the nominal pH 8.25 data.

The data in Figure 3.2 and 3.3 include values for all of the different water hardnesses. Although it cannot be seen explicitly from these figures, the hardness has no systematic effect on Al(III) concentrations, as would be expected.

Although the solubility constant for crystalline gibbsite and formation constants for some of the simple hydrolysis species (e.g., $Al(OH)_4^-$) appear to be fairly well established, there is still lack of unanimity over equilibrium constants for most of the other species (Apps and Neil, 1990 ; May et al., 1979). In this study we shall not elaborate on these controversies, but simply attempt to show where the solutions in this studies stand with respect to solutions saturated with " a typical non-highly crystalline" $Al(OH)_3$ (s). Three types of $Al(OH)_3$ (s) were reported by Nordstrom et al., 1990, namely, "amorphous" $Al(OH)_3$ (s), "microcrystalline gibbsite", and crystalline gibbsite. These three phases represent a likely value and the extrema to be expected for $Al(OH)_3$ (s) in this study. Solubility constants (Nordstrom et al., 1990) and complex formation constants (Nordstrom et al., 1990) are given in Table 3.4

In Figure 3.4 show the speciation calculated for a solution in equilibrium with microcrystalline gibbsite. The usual polynuclear species that are reported are

significant contributors to the total soluble Al(III) only at pH values below 6.0, that is, outside of the range of this study, and thus are included. The significant species for this study is primarily $Al(OH)_4^-$, and $Al(OH)_3$ (aq), the formation constant of which is open to question.

Also shown in Figure 3.4 are the filterable Al(III)-pH data for both experiments. The data for the nominal pH 7.25 experiment, Trial I (which showed the equilibrium-like independence of total Al(III) in Figure 3.2) does indeed approach the equilibrium line in Figure 3.4. The data for the nominal pH 7.25 experiment, Trial II, and the nominal pH 8.25 experiment show values that are greater than the solubility limit and quite simply completely inconsistent with any kind of a simple solubility equilibrium model.

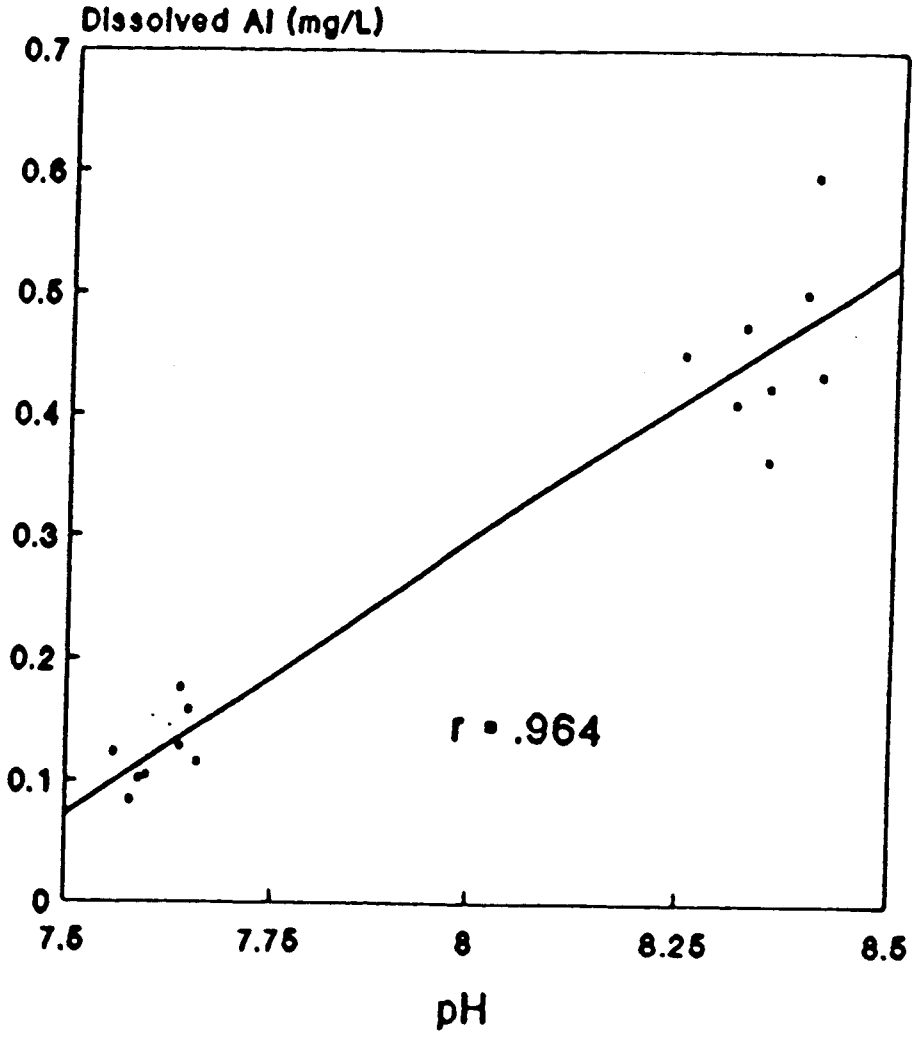



Figure 3.1. Solubility of aluminum as a function of pH at total concentrations of Al = 4 ppm.

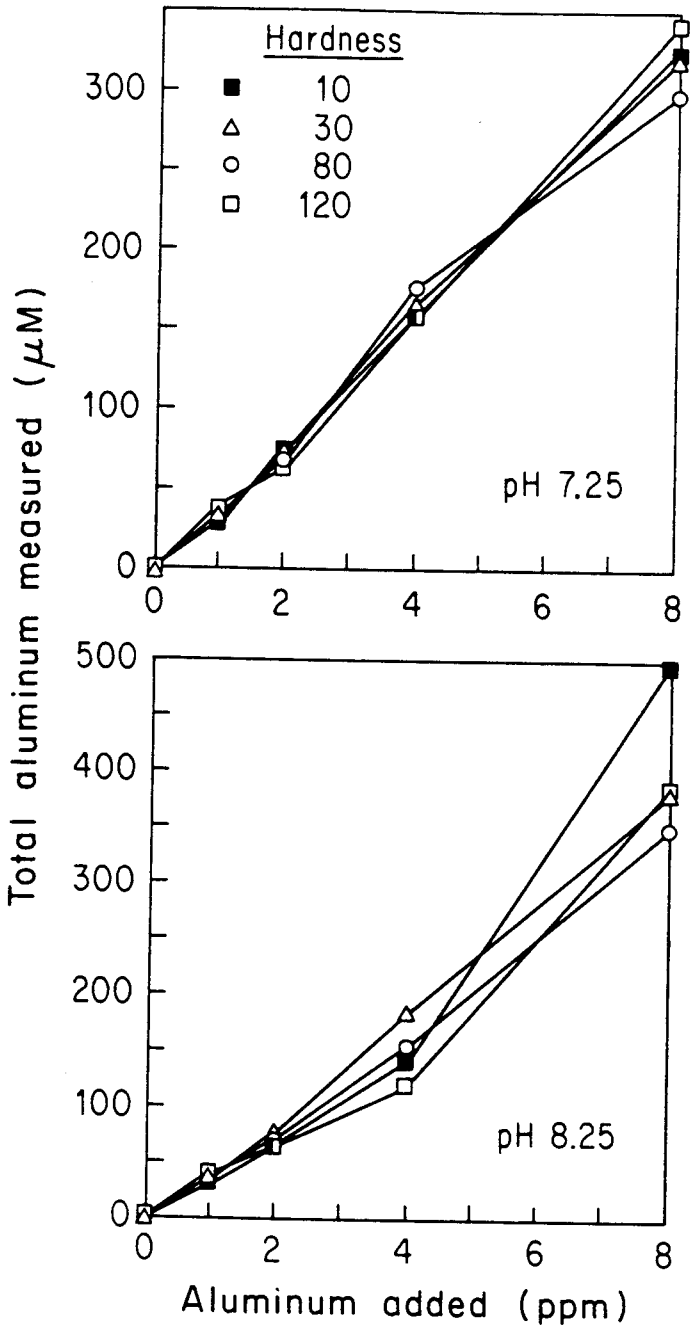



Figure 3.2. The relationship between total Al(III) measured ($\mu$M) and total Al(III) added (ppm) with various hardness at nominal pH 7.25 and pH 8.25.

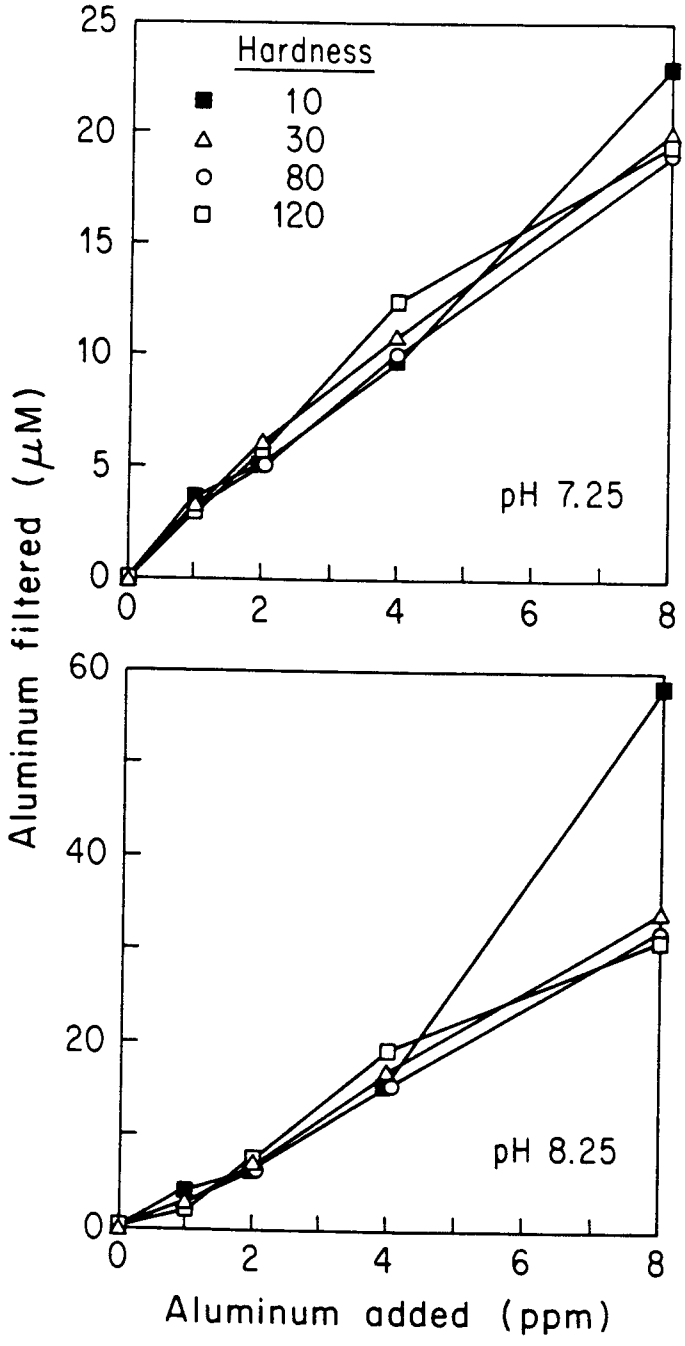



Figure 3.3. The relationship between Al(III) filtered ($\mu$M) and total Al(III) added (ppm) with various hardness at nominal pH 7.25 and pH 8.25.

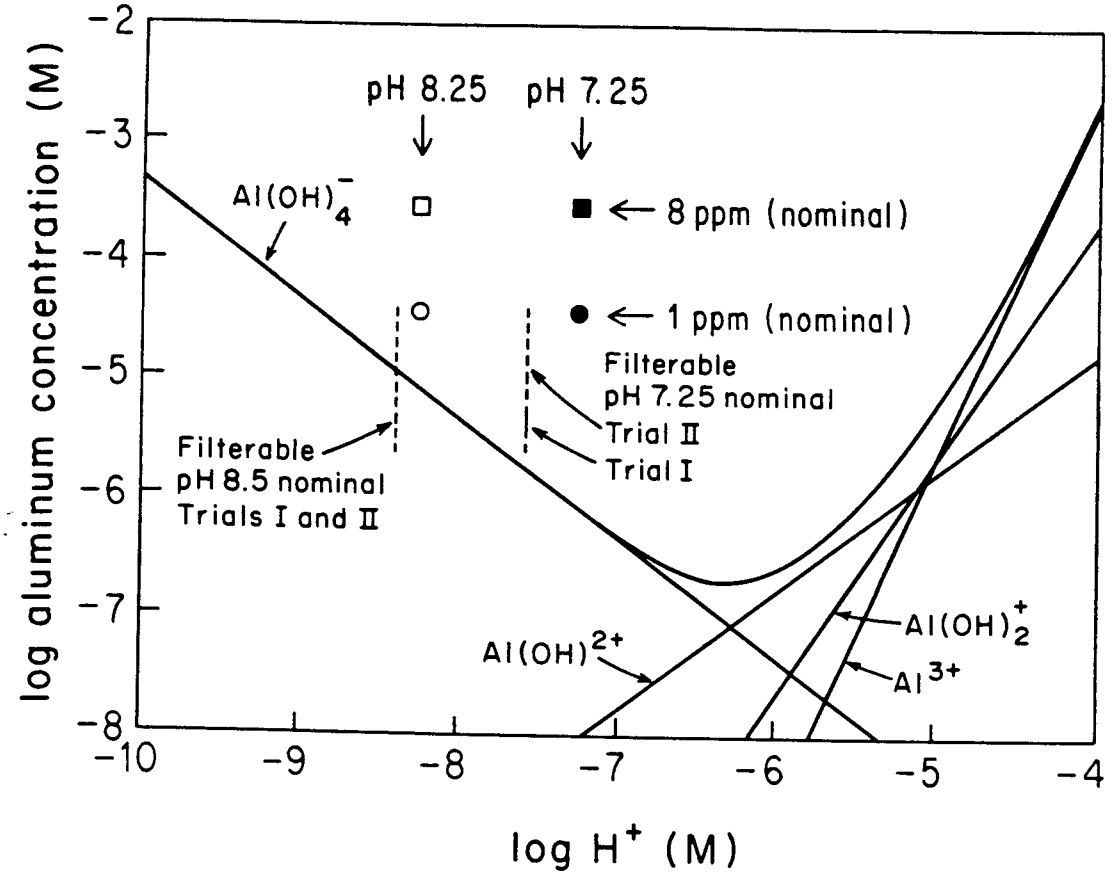



Figure 3.4. The solubility and species of aluminum.

Table 3.1. Average pH, total hardness and measured aluminum for two test chambers, LC50 calculated at nominal pH = 8.25 (alkaline pH).

| Variable | Average pH | Total hardness (ppm $CaCO_3$) | Measured aluminum concentration (ppm) | | | |
|---|---|---|---|---|---|---|
| | | | dissolved | | total | |
| | | | high | low | high | low |
| 10/4 | 8.34 | 23.44 | 0.473 | 0.363 | 5.51 | 2.84 |
| 10/8 | 8.58 | 24.63 | 2.210 | 0.948 | 14.04 | 12.69 |
| 30/4 | 8.37 | 36.31 | 0.501 | 0.424 | 7.04 | 2.99 |
| 30/8 | 8.59 | 37.74 | 0.936 | 0.935 | 11.93 | 8.68 |
| 80/4 | 8.36 | 82.93 | 0.434 | 0.411 | 4.25 | 4.09 |
| 80/8 | 8.56 | 83.34 | 0.915 | 0.840 | 11.65 | 7.16 |
| 120/4 | 8.33 | 118.46 | 0.957 | 0.450 | 4.42 | 3.47 |
| 120/8 | 8.56 | 120.24 | 0.852 | 0.840 | 10.53 | 10.11 |

Table 3.2. Average pH, total hardness and measured aluminum for two test chambers, LC50 calculated at nominal pH = 7.25 (neutral pH).

| Variable | Average pH | Total hardness (ppm $CaCO_3$) | Measured aluminum concentration (ppm) | | | |
|---|---|---|---|---|---|---|
| | | | dissolved | | total | |
| | | | high | low | high | low |
| 10/4 | 7.63 | 25.29 | 0.417 | 0.109 | 4.87 | 3.73 |
| 10/8 | 7.59 | 27.35 | 1.000 | 0.121 | 9.85 | 7.81 |
| 30/4 | 7.62 | 44.65 | 0.462 | 0.130 | 5.60 | 4.06 |
| 30/8 | 7.62 | 45.24 | 0.936 | 0.199 | 9.33 | 8.07 |
| 80/4 | 7.61 | 86.91 | 0.422 | 0.124 | 5.36 | 4.17 |
| 80/8 | 7.58 | 89.51 | 0.877 | 0.149 | 8.16 | 7.95 |
| 120/4 | 7.60 | 127.98 | 0.523 | 0.149 | 8.16 | 7.95 |
| 120/8 | 7.59 | 130.38 | 0.896 | 0.158 | 10.30 | 8.20 |

Table 3.3. Al(III) species in natural waters.

| |
|---|
| Hydroxide species |
| mononuclear complexes (e.g., $Al^{3+}$, $[Al(OH)]^{2+}$, $[Al(OH)_2]^{+}$, $[Al(OH)_4]^{-}$ |
| simple polynuclear (e.g., $[Al_3(OH)_4]^{5+}$, $[Al_{13}O_4(OH)_{24}]^{7+}$ |
| polymeric |
| colloidal particles |
| amorphous solids |
| microcrystalline solids |
| crystalline solids |
| Other Inorganic Complexes |
| complexes with simple inorganic ligands (e.g., $F^{-}$, $SO_4^{2-}$ ) |
| Organic complexes |
| complexes with simple organic ligands (e.g., oxalate) |
| complexes with complex organic polymers (e.g., mucous) |

Table 3.4. Equilibrium constant (I = 0, T = 298 K) from Nordstrom et al., 1990.

| Reaction | log K |
|---|---|
| $Al^{3+} + H_2O = Al(OH)^+ + H^+$ | -5.01 |
| $Al^{3+} + 2\ H_2O = Al(OH)_2^+ + H^+$ | -10.12 |
| $Al^{3+} + 3\ H_2O = Al(OH)_3\ (aq) + 3\ H^+$ | -16.95 |
| $Al^{3+} + 4\ H_2O = Al(OH)_4^- + 4\ H^+$ | -22.68 |
| $Al(OH)_3$ (crystalline gibbsite) + $3H^+ = Al^{3+} + 3\ H_2O$ | 8.11 |
| $Al(OH)_3$ (microcrystalline gibbsite) + $3H^+ = Al^{3+} + 3\ H_2O$ | 9.35 |
| $Al(OH)_3$ (amorphous) + $3\ H^+ = Al^{3+} + 3\ H_2O$ | 10.8 |

## 3.1 Acute Toxicity

The effect of water hardness and pH on the toxicity of aluminum to rainbow trout can be demonstrated by examination of the 96 hr LC50 determinations for aluminum at each hardness and pH tested. Table 3.5 lists the median lethal concentration values for LC50, based upon measured aluminum concentrations and calculated by the Trimmed Spearman-Karber (Hamilton et al., 1971).

Table 3.5. Influence of water hardness on acute aluminum toxicity (LC50 at 96 hr) in rainbow trout using measured $Al^{+3}$ concentration for neutral and alkaline pH.

| Nominal pH | Nominal hardness | LC50[a] based on measured aluminum | | | |
|---|---|---|---|---|---|
| | | dissolved | | total | |
| | | 1[b] | 2 | 1 | 2 |
| **7.25** | 10 | >0.12* | >1.0* | >9.85* | >7.81* |
| | 30 | >0.20* | >0.94* | >8.07* | >9.33* |
| | 80 | >0.15* | >0.88* | >8.16* | >7.95* |
| | 120 | >0.16* | >0.90* | >8.20* | >10.30* |
| 8.25 | 10 | 0.59 | 0.53 | 6.81 | 6.09 |
| | | (0.5-0.7) | (0.4-0.6) | (5.5-8.5) | (5.2-7.1) |
| | 30 | 0.52 | 0.54 | 6.84 | 6.58 |
| | | (0.4-0.6) | (0.4-0.7) | (5.8-8.0) | (5.4-8.0) |
| | 80 | 0.69 | 0.73 | 7.33 | 7.67 |
| | | (NR[c]) | (0.6-0.9) | (5.4-9.9) | (6.2-9.5) |
| | 120 | 0.73 | 0.74 | 7.41 | 7.54 |
| | | (NR) | (NR) | (NR) | (NR) |

[a]LC50 results are from Spearman-Karber analysis, 95% confidence levels in parentheses; [b]replicates; [c]not reliable.*no mortality at highest concentration tested

The effects of the hardness tests on the toxicity of aluminum to rainbow trout indicate that there was a correspondingly lower resistance to aluminum toxicity over the entire range of pH at lower values of hardness. Table 3.6 shows the results of the ANOVA, based on Petersen (1985), for the eight LC50 values for each group, readily demonstrating the effect of water hardness upon aluminum toxicity among rainbow trout.

Table 3.6. One-way analysis of variance for LC50, hardness at nominal pH = 8.25.

| Source of variation | F-value | Sign. level |
|---|---|---|
| LC50 dissolved | 30.193 | .0033 |
| LC50 total | 6.083 | .0568 |

Statistical tests could not be conducted for LC50s with either dissolved or total aluminum at pH 7.25 since definitive values could not be calculated.

At a nominal pH of 7.25 (mean mesured pH = 7.61), the concentrations of aluminum were not sufficient to kill any fish. Therefore no differences in mortality at different water hardness could be measured. The mortality data at nominal pH 8.25 (mean measured pH = 8.27) are presented in Table 3.8. Figure 3.5 shows the percentage of fish which survived following exposure for 96 hr at varying degrees of water hardness and different levels of pH.

Table 3.7. Cumulative mortalities[a] of rainbow trout exposed to aluminum solution for various degrees of hardness, nominal pH = 7.25.

| Exposure time (hrs) | Nominal hardness | Nominal aluminum concentrations (ppm) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 0 | 0 | 1 | 1 | 2 | 2 | 4 | 4 | 8 | 8 |
| | | a | b | a | b | a | b | a | b | a | b |
| 24 | 10 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 30 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 80 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 120 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 48 | 10 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 30 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 80 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 120 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 72 | 10 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 30 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 80 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 120 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 96 | 10 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 30 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 80 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 120 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

[a]Ten fish per replicated were tested; columns a and b are replicates.

Table 3.8. Cumulative mortalities[a] for rainbow trout exposed to aluminum solution at various degrees of hardness, nominal pH = 8.25.

| Exposure time (hrs) | Nominal hardness | Nominal aluminum concentrations (ppm) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 0 | 0 | 1 | 1 | 2 | 2 | 4 | 4 | 8 | 8 |
| | | a | b | a | b | a | b | a | b | a | b |
| 24 | 10 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 6 |
| | 30 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 4 | 5 |
| | 80 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| | 120 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 3 | 1 |
| 48 | 10 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 9 | 9 |
| | 30 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 3 | 7 | 9 |
| | 80 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 4 | 2 |
| | 120 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 6 | 6 |
| 72 | 10 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 10 | 10 |
| | 30 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 3 | 9 | 10 |
| | 80 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 7 | 4 |
| | 120 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 8 | 7 |
| 96 | 10 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 1 | 10 | 10 |
| | 30 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 3 | 9 | 10 |
| | 80 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 8 | 5 |
| | 120 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 9 | 8 |

[a]Ten fish per replicate were tested; columns a and b are replicates.

Table 3.9. Aluminum concentrations of gill tissue after 96 hour exposure at various degrees of hardness, nominal pH 7.25.

| Hardness/Aluminum[a] | Al ($\mu g\ g^{-1}$ wet weight ) | |
|---|---|---|
| | I | II |
| 10/0 | 0.75 | 0.84 |
| 30/0 | 0.67 | 0.85 |
| 80/0 | 0.76 | 0.79 |
| 120/0 | 0.95 | 0.82 |
| 10/1 | 7.33 | 4.04 |
| 30/1 | 9.08 | 4.11 |
| 80/1 | 2.13 | 4.11 |
| 120/1 | 1.86 | 4.47 |
| 10/2 | 10.02 | 6.43 |
| 30/2 | 10.43 | 5.99 |
| 80/2 | 13.77 | 4.28 |
| 120/2 | 7.63 | 4.31 |
| 10/4 | 8.06 | 5.87 |
| 30/4 | 10.49 | 5.54 |
| 80/4 | 14.78 | 5.24 |
| 120/4 | 16.03 | 5.75 |
| 10/8 | 23.46 | 5.02 |
| 30/8 | 11.45 | 6.92 |
| 80/8 | 7.64 | 7.66 |
| 120/8 | 7.95 | 5.75 |

Columns (I) and (II) are for replicated experiments.

[a]. Hardness is as mg $CaCO_3$/L and aluminum is total (mg/L) added (nominal).

Table 3.10. Aluminum concentrations of gill tissue after 24, 48, 72 hour exposure at various degrees of hardness, nominal Al concentration = 8 ppm and pH 8.25.

| Exposure time (hrs) | Hardness/Aluminum[a] | Al ($\mu g\ g^{-1}$ wet weight) | |
|---|---|---|---|
| | | (I) | (II) |
| 24 | 10/8 | 33.95 | 25.28 |
| | 30/8 | 53.22 | 34.29 |
| | 80/8 | 50.69 | 92.36 |
| | 120/8 | 51.34 | 64.10 |
| 48 | 10/8 | 88.73 | 53.18 |
| | 30/8 | 80.18 | 59.85 |
| | 80/8 | 95.48 | 92.36 |
| | 120/8 | 83.54 | 77.12 |
| 72 | 10/8 | 183.64 | 119.37 |
| | 30/8 | 109.27 | 202.61 |
| | 80/8 | 100.62 | 123.24 |
| | 120/8 | 82.88 | 148.55 |

Columns (I) and (II) are for replicated experiments.

[a]. Hardness is as mg $CaCO_3$/L and aluminum is total (mg/L) added (nominal).

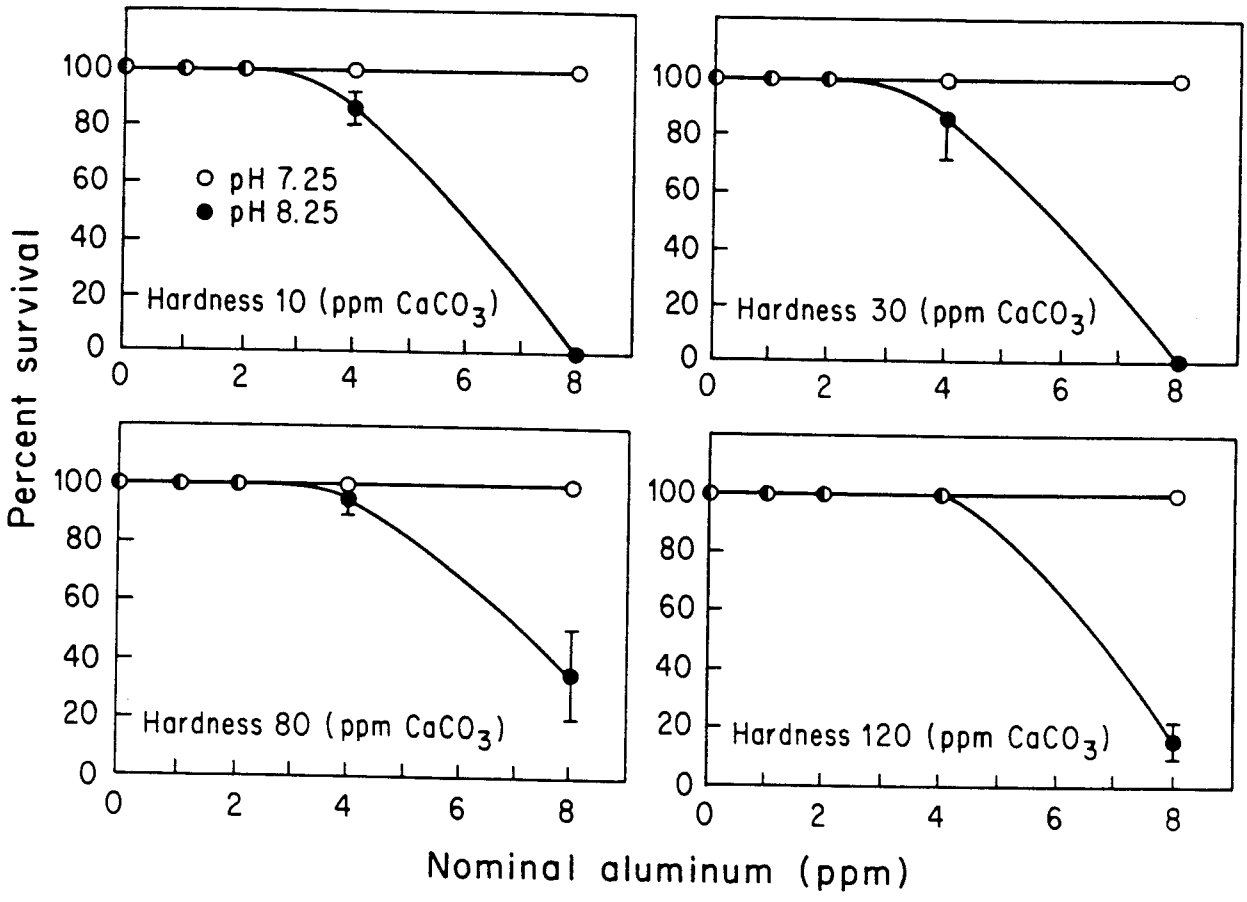



Figure 3.5. Survival of rainbow trout following exposure for 96 hr in various degrees of water hardness for different levels of pH.

## 3.2 Aluminum Accumulation in Gill Tissue.

The accumulation of aluminum in the gills of fish surviving 96 hrs of exposure at a nominal pH of 7.25 was between 0.8 and 23.5 $\mu g.g^{-1}$ wet weight. A two-way ANOVA for the accumulation of aluminum indicated that there were no significant effects for water hardness on gill accumulation (F-value = .246, Sig. level = .8631), but that there was a highly significant effect of aluminum concentration (F-value = 5.453, Sig. level = .0039).

At a nominal pH of 8.25, aluminum was detected in the gills of dead fish following exposures of 24, 48, 72, and 96 hr in accumulations from 25 to 200 $\mu g.g^{-1}$ of wet tissue. For the dead fish, the two-way ANOVA for gill accumulation showed that there was no significant effect for water hardness (F-value = .307, Sig. level = .8199), but that there was a highly significant effect for exposure times (F-value = 15.661, Sig. level = .0005). Because different sample fish were used (dead fish at pH 8.25 and as live fish at pH 7.25) statistical tests could not be performed for the effect of changes in pH on gill accumulation at the same degrees of hardness (Figure 3.6).

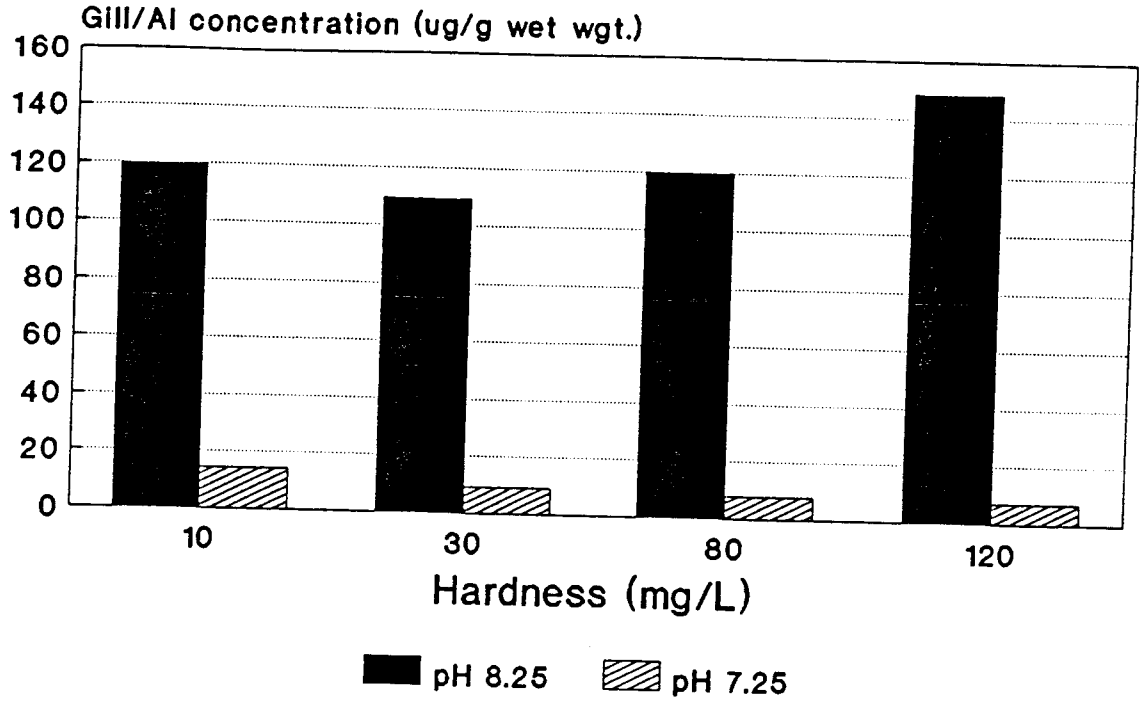



Figure 3.6. Aluminum concentrations of gill tissue after 96 hour exposures to a nominal concentration of 8 ppm.

# 4. DISCUSSION

## 4.1 Acute Toxicity

The results of this investigation verified greater acute toxicity of aluminum at 8.25 compared to 7.25 is associated with higher dissolved aluminum concentrations at alkaline than near neutral pH. The experiments revealed no aluminum-induced mortality among rainbow trout at a nominal pH of 7.25 (Table 3.7). However, at a nominal pH of 8.25 (Table 3.8), rates of mortality among rainbow trout sharply increased with dissolved aluminum concentration. This finding confirms evidence presented by Freeman and Everhart (1971), who determined that onset of aluminum toxicity increased rapidly as pH levels are increased and suggested that soluble aluminum is an acutely toxic metallic form.

In tests at near neutral pH values (i.e., a measured pH of 7.6), aluminum was not found to be acutely toxic, even at a nominal aluminum concentration of 8 ppm. However, Call et al. (1984) reported a 96 hr LC50 for rainbow trout at a nominal pH of 7.5 (mean measured pH = 7.31) of 14.6 (9.3—23.1) ppm , whereas Freeman and Everhart (1971) had previously found that total aluminum concentra-

tions as low as 0.52 ppm caused mortality among trout starting in the fourth week of the test at a pH of 7.0.

Interpretation of the toxicological effects of aluminum is complicated by the presence of a variety of chemical species. The effect of pH on the toxicity of aluminum may be explained by aluminum speciation. Dissolved aluminum was estimated by filtration and no detailed analyses of speciation for these experiments are available.

Our conclusions about the solution speciation of Al(III) are:

1. One experiment, nominal pH 7.25, Trial I, is reasonably close to equilibrium behavior: the solubility product for the filterable fraction is reasonably close to that expected for a poorly crystallized alumina, and the Al(III) in the filterable fraction is relatively independent of the total Al(III) in the system.

2. In all other experiments, the solutions appeared to be extremely supersaturated: the amount of Al(III) in the filterable fraction is roughly 10 % of the total , it still exceeds the solubility, and it is probably composed of polymeric colloidal Al(III) - OH species or organic complexes.

3. The differences in behavior of these solutions raise important questions for the toxicity tests. Mortality occurs at pH 8.25 with greater than about 150 $\mu$M toatl Al(III) or 15 $\mu$M filterable Al(III). If the differences between the nominal pH 7.25 Trial I and the other

experiments are trough, the other experiments may be in transition from the toxic condition to the sub-toxic condition (i.e., transition from high Al(III) to low Al(III)). Thus variables such as retention time of the Al(III) solutions or the availability of small particles as nucleation sites might be determining parameters for Al(III) levels and of toxicity. Thus careful monitoring of the evolution of the Al(III) hydroxides is indicated.

4. At this point, we cannot reach any conclusion about effect of various Al(III) species on toxicity; effectively the same levels of total and filterable Al(III) were in solution at pH 7.6 and pH 8.3, yet the pH 8.3 solution killed the fish and the pH 7.6 solution did not. Thus, from the point of view of regulations, we can do no better to propose as a working hypothesis that regulations be based on total Al(III) (since it is simpler to determine), pH, and hardness; however, we feel that there is certainly more imformation to be gained from controlled studies of the colloidal forms, and that the recommendation for regulation based on total Al(III) is just a temporary measure until controlled experiments with colloidal Al(III) can be carried out.

In tests at pH 7.25 (neutral) and 8.25 (alkaline), it is possible that solid $Al(OH)_3$ and $Al(OH)_4^-$ are formed, with solid $Al(OH)_3$ predominant at pH 7.25 and soluble $Al(OH)_4^-$ predominant at pH 8.25. Indeed, at pH 7.6 small amounts of dissolved aluminum were found (Fig. 3.4) since solid

$Al(OH)_3$ is predominant. Two distinct mechanisms for aluminum toxicity are deposition of solid aluminum on gill and ionic interaction of soluble species (Booth et al., 1988). It is possible that the precipitation of soluble monomeric aluminum species into either polymers and/or other solids at the gill surface could result in gill damage and osmoregulatory disturbances (Schofield and Trojnar, 1980, cited in Booth et al., 1988). Differences in toxicity among aluminum species can be found at certain pH values, and both toxic mechanisms may actively produce gill damage, with one of the two predominant under specific conditions.

In this investigation, there was no evidence of acute mortality among rainbow trout exposed for 96 hr to aluminum at pH 7.25. Therefore, the effect of water hardness on the acute toxicity of aluminum to rainbow trout cannot be examined under these conditons. Tests conducted at pH 8.25, indicate that at lower hardness there was a correspondingly lower resistance to aluminum toxicity. At hardness levels of 10 and 30 ppm of $CaCO_3$ there was no difference in aluminum toxicity (Table 3.8). When water hardness increased to 80 ppm of $CaCO_3$ toxicity was reduced. In fact, $Ca^{2+}$ provided a significant protective effect (i.e., Table 3.6, $p < 0.05$). Booth et al. (1988) also found that at acidic pH values (i.e. 4.4—5.2), $Ca^{2+}$ protected against aluminum-induced ion loss, thereby reducing mortality among brook trout. In low-$Ca^{2+}$ waters, various

combinations of aluminum and pH values contributed to overall mortality of 35 percent (i.e., 19 fish of a total of 54). In contrast, the mortality rate in high-$Ca^{2+}$ waters was only 13 percent (7 fish out of a total of 54).

The biological action of $Ca^{2+}$ is dependent upon its ability to bind to proteins, and is therefore related to its basic chemical properties. Calcium binds to the surface of the gills, and this binding is essential for the maintenance of the ionic and osmotic gill regulatory functions. Competition for binding sites on the gills between $Ca^{2+}$ and other divalent or trivalent metallic ions exercises an influence upon metal uptake and their degree of toxicity to fish (Hunn, 1985). By reducing the loss of ions, $Ca^{2+}$ offers a degree of protection and thereby serves to reduce fish mortality. The ionic interactions between $Ca^{2+}$ and surface ligands act to stabilize the apical membranes of the gill epithelium, thereby increasing the tightness of the intercellular gill junctions (McDonald, 1983), reducing the permeability of the membrane, and increasing its resistance to attack by surface-active toxicants. Furthermore, the result of increasing water $Ca^{2+}$ may be to delay or reduce the binding of aluminum at these sites. Alternatively, since neither of the processes in question require prior binding of aluminum to gill anions, high $Ca^{2+}$ in water may promote the polymerization or the precipitation of aluminum on the surface of the gills.

## 4.2 Aluminum Accumulation in Gill Tissue

The experimental results indicate that the accumulation of aluminum in gill tissues at pH 8.25 is higher than for pH 7.25 following 96 hr of exposure (Figure 3.6). This finding may be explained as follows for such gill contaminants as aluminum, the toxicities of which vary with the level of pH: In comparison to bulk-inspired waters, higher or lower pH values near the gills may change toxicant solubility or speciation in the branchial micro-environment. Aluminum is thus precipitated onto the gills when the pH value of the inspired water is below approximately 5.7 because of the more basic conditions near the gills (Playle and Wood, 1989). If inspired water passes across the gills the changes in water condition will reflect, for example, a change from an acidic to a more basic media or, to the contrary, from basic to more acidic media (Randall and Wright, 1989). The changes which take place between water inspiration and expiration can be explained by the release of carbon dioxide and ammonia at the gills, which tend to respectively acidify or to alkalinize water passing over the gills. Thus, changes in aluminum speciation are important factors with respect to aluminum deposition upon fish. Note that Playle and Wood (1989) reported that increased inspiration of pH followed increased aluminum accumulation on the gills, providing a curve which is

identical with the aluminum solubility curve used for microcrystalline gibbsites (Roberson and Hem, 1969).

The relative contributions of polymerization, adsorption, and complexation to gill aluminum deposition is influenced by such factors as the pH value of the gill microenvironment, aluminum concentration, solution ionic strength and buffer capacity, the existence of a negative charge in the mucous and on the gill surface, and the amount of mucous produced at the gills and on the gill surface area. Increasing aluminum concentrations from 0 to 8 ppm resulted in an increase of aluminum accumulation at the gills (Table 3.9), where these concentrations had a significantly cumulative effect ($p < 0.05$). The rate at which the gills of rainbow trout will accumulate a toxicant is dependent upon its concentration in the water, the affinity of the toxicant for gill and the rate at which water moves through the channels between the secondary lamellae (Weber, 1984). Statistical testing at $p < 0.05$ has shown that exposure time has a significant effect (Table 3.10). The differences in accumulated toxicant in the gills after immersion may be explained by the contact time effect of the toxicant.

From the experiments conducted for this investigation, no significant effects of water hardness on aluminum accumulation by the gills could be determined for pH 7.25 and 8.25 ($p > 0.05$). However, McDonald and Wood (unpublished results cited in Playle et al., 1989) reported that

$Ca^{2+}$ reduces aluminum accumulation at the gills. In addition, brook trout yolk-sac fry and swim-up fry, at pH values of 4.8 and 5.2, accumulated less aluminum in waters with higher $Ca^{2+}$ than in waters with lower $Ca^{2+}$ (Wood et al., 1990a, 1990b). Only a small amount of aluminum precipitation is required to cause gill inflammation and thereby ion loss, and, owing to cell swelling and mucous accumulation, larger amounts of precipitated aluminum are necessary to impair the increased diffusion distance of the gas transfer trough (Playle et al. 1989).

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